Study Details

Test No. 2017338
Product Name PI 1525

Cas-No:	EC-No:	Chemical Name:
1897392-68-5		4,7-Methano-1H-indene, 5-ethoxyoctahydro-,(3aR,4R,5S,7R,7aR)-rel-

Product code	672994
Product Name	PI 1525
Test code	PI 1525
Purity	95,3 (if 0,0 then see remarks)
Batch No.	Ho 154 262 MM + 0.1% Vit.E
Study code	DAI17197
Institute Name	NOACK LABORATORIEN GmbH
Description	Daphnia sp. Acute Immobilisation Test, OECD 202, EU C.2
Final Report date	20.02.2018
Results	EC50 (48 h) = 1.90 mg/L (geometric mean of measured concentration); Daphnia magna; Semi-static, closed system without headspace
Reliability	Rel 1
GLP	YES
Remark	Rel.1: according to OECD 202 (2004) and GLP

- Report -

PI 1525

Acute Immobilization Test to *Daphnia magna*, Semi-static, 48 hours, in a Closed System without Headspace

acc. to OECD-Guideline 202 for Testing of Chemicals (2004)

Sponsor

Author

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Study ID
acc. to GLP

170927SY / DAI17197

Study completed on

20 FEB 2018

Statement of GLP Compliance

Title	PI 1525 Acute Immobilization Test to *Daphnia magna*, Semi-static, 48 hours, in a Closed System without Headspace
Guideline	OECD-Guideline 202 for Testing of Chemicals (2004)
Test Item	PI 1525 (batch number: Ho154262-MM +0.1% Vit.E.)
Test Facility	Noack Laboratorien GmbH Käthe-Paulus-Str.1, 31157 Sarstedt, Germany Phone: +49 5066 7067 0, Fax: +49 5066 7067 89 E-mail: info@noack-lab.de

We declare that this study was conducted and reported in compliance with the present OECD, EC and German principles of Good Laboratory Practice.

20.2.18 .....
(Date) (Dirk Scheerbaum, Study Director)

20.2.18 .....
(Date) (Karin Petersen, Scientist - Analytical Department)

Statement of the Quality Assurance Unit

Title	PI 1525 Acute Immobilization Test to *Daphnia magna*, Semi-static, 48 hours, in a Closed System without Headspace
Guidelines	OECD-Guideline 202 for Testing of Chemicals (2004)
Test Item	PI 1525 (batch number: Ho154262-MM +0.1% Vit.E.)
Study Director	Dirk Scheerbaum

The study was verified and reported to the study director and test facility management as follows.

Inspected study phase		Inspection date	Date of report
Study plan		2017-10-26 2017-11-03	2017-10-26 2017-11-03
Experimental phase	Observation(s), Measurement(s)	2017-11-16	2017-11-16
Report		2017-12-08 2017-12-12 2017-12-18 2018-02-20	2017-12-08 2017-12-12 2017-12-18 2018-02-20

The reported results accurately and completely reflect the raw data of the study. Also methods, procedures and observations are accurately and completely described in the report.
The accordance of the study with its study plan and the principles of Good Laboratory Practice is guaranteed.

20.02.2018 .....
(Date) (Dr. Bianca von Thülen, QAU)

Personnel Involved

Study Director:	Dirk Scheerbaum (Biologist)
Scientist:	Karin Petersen (Food chemist) Responsible for the analytical monitoring
Technical Staff:	Alexandra Donath Monika König Thomas Nowakowski Katharina Warnecke
Quality Assurance Unit:	Gudrun Möhrmann-Kalabokidis (Head of QAU, Biologist) Christine Bruhnke (Biologist) Dr. Bianca von Thülen (Biologist)
Test Facility Management:	Dr. Christian Maeß (Chemist)

Table of Contents

List of Tables

List of Figures

1 List of Abbreviations / Definitions

CI	Confidence Interval, expressed as 95% Confidence Limits
CV	Coefficient of Variation
Date format	YYYY-MM-DD (Year-Month-Day)
$EC_{10 / 50 / 100}$	The concentration of the test item / reference item estimated to result in a 10 / 50 / 100% immobilization rate
(ID) x (H)	Inner Diameter x Height
GC-MS	Gas Chromatography-Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
lx	Lux; i.e. the SI unit of illuminance and luminous emittance, measuring luminous flux per unit area
MV	Mean Value
QAU	Quality Assurance Unit
R^2	Coefficient of Determination
rpm	Revolutions Per Minute
Saturated solution	The maximum dissolved concentration of the test item that can be achieved under the test conditions in the test medium, acc. to OECD Series, No. 23 (2000).
SD	Standard Deviation
S/N	Signal to Noise Ratio

2 Summary

In the acute immobilization test with *Daphnia magna* (STRAUS), the effects of the test item PI 1525 (batch number: Ho154262-MM +0.1% Vit.E.) were determined at the test facility according to OECD 202 (2004) from 2017-11-15 to 2017-11-23, with the definitive exposure phase from 2017-11-15 to 2017-11-17.

The study was conducted in a closed system (sealed glass flasks) without headspace under semi-static conditions over a period of 48 hours with the undiluted saturated solution of the test item and further five dilution levels (nominal: 1.94 to 100%) prepared from the saturated solution in a geometric series with a separation factor of 2.2.

Twenty daphnids (divided into 4 replicates with 5 daphnids each) were exposed to each concentration level and the control.

The concentrations of the test item were analytically verified via GC-MS in the fresh media at the start of the exposure and at the renewal of the test solutions (0 and 24 hours) and in the old media at the renewal and at the end of the test (24 and 48 hours) in all concentration levels and in the control. Details of the analytical method are presented in section 14. Results of the method validation are presented in Annex I.

The measured concentrations in the old media at the renewal and at the end of the test (24 and 48 hours) were in the range of 63 to 128% of the initially measured concentrations. The geometric mean measured concentrations are: 0.415 – 0.811 – 1.69 – 4.24 – 8.42 – 13.4 mg/L. The analytical results are presented in Table 5.

The effect concentrations given in Table 1 are based on the geometric mean measured concentrations of the test item PI 1525.

The validity criteria of the test guideline were fulfilled.

Table 1: **EC_{10}-, EC_{50}- (with 95% Confidence Limits) and EC_{100}-Values**
(based on the geometric mean measured concentrations of the test item)

PI 1525		
Effect concentrations	**Test duration [hours]**	**Geometric mean measured test item concentrations [mg/L]**
EC_{10} (with 95% confidence limits)	24	8.02 (CI: 4.24 – 13.4)
	48	0.939 (CI: 0.557 – 1.29)
EC_{50} (with 95% confidence limits)	24	10.1 (CI: 4.24 – 13.4)
	48	1.90 (CI: 1.55 – 2.49)
EC_{100}	24	13.4
	48	8.42

3 Characterization Data of the Test Item

3.1 Test Item Properties

Test Item	PI 1525
Batch number	Ho154262-MM +0.1% Vit.E.
Chemical name	Mixture of 4,7-Methano-1H-indene, 5-ethoxyoctahydro-, (3aR,4R,5S,7R,7aR)-rel- and 4,7-Methano-1H-indene, 5-ethoxyoctahydro-,(3aR,4S,5R,7S,7aR)-rel-
CAS number	1) 1897392-68-5 and 2) 13213-08-6
Molecular formula	$C_{12}H_{20}O$
Molecular weight	180 g/mol
Purity (certified)	95.3 % 1) Constituent 1: 4,7-Methano-1H-indene, 5-ethoxyoctahydro-, (3aR,4R,5S,7R,7aR)-rel, ca. 83% 2) Constituent 2: 4,7-Methano-1H-indene, 5-ethoxyoctahydro-, (3aR,4S,5R,7S,7aR)-rel-, ca. 12%
Appearance	Clear, colorless liquid
Water solubility	45.9 mg/L at 20 °C and pH 6.7
Vapor pressure	< 1 kPa at 50°C (calculated)
Log P_{OW}	4.021 at 25 °C and pH 5.59
Stability under test conditions	Not specified
Expiry date	2017-11-30
Recommended storage	Dry, ambient temperature in the tightly closed original container, away from moisture, heat and light

The test item and the information concerning the test item were provided by the sponsor.

3.2 Test Facility Actions

Receipt	2016-06-22
Identification parameter	Name, batch number, state, color and turbidity
Retention sample	At least 1 g has been sampled on 2016-06-30 and will be retained at 6 ± 2 °C.
Storage conditions	Room temperature, protected from light in the tightly closed original container

4 Method

Test guidelines	• OECD-Guideline 202 for Testing of Chemicals (2004), "*Daphnia* sp., Acute Immobilization Test" and under consideration of • OECD series on testing and assessment no. 23, ENV/JM/MONO(2000)6 The study was performed in compliance with GLP. For the respective guidelines, please refer to section 10.
Type and purpose of the study	An acute immobilization test to *Daphnia magna* STRAUS was carried out to determine the $EC_{10/50/100}$-values of the test item after 24 and 48 hours of exposure under semi-static conditions in a closed system without headspace.

4.1 Test System and Culture

Test system	*Daphnia magna* STRAUS (Clone 5)
Reason for the selection of the test system	*Daphnia magna* is the preferred species in accordance with the test guideline and is bred at the test facility.
Origin	Institut für Wasser-, Boden- und Lufthygiene (WaBoLu), 14195 Berlin, Germany
Breeder	Noack Laboratorien GmbH, Käthe-Paulus-Str. 1, 31157 Sarstedt, Germany
Culture	In glass vessels (2 - 3 L capacity) with approximately 1.8 L culture medium, at 20 ± 2 °C, in an incubator, 16 hours illumination, light intensity of max. 1500 lx
Culture medium	Elendt M4, according to OECD 202, Annex 3 (2004), modified to a total hardness of 160 to 180 mg $CaCO_3$/L, is used. The composition of the culture medium is presented in Table 2.
Feeding of the culture stocks	The daphnids are fed at least 5 times per week *ad libitum* with a mix of unicellular green algae, e.g. *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus,* with a cell density of > 10^6 cells/mL. The algae are cultured at the test facility.
Origin of the food algae	Sammlung von Algenkulturen (SAG), Pflanzenphysiologisches Institut der Universität Göttingen, Nikolausberger Weg 18, 37073 Göttingen, Germany

Table 2: **Composition of the Culture Medium Elendt M4** according to OECD 202, Annex 3 (2004)

Component	Concentration [mg/L]
$CaCl_2 \times 2\ H_2O$	176*
$MgSO_4 \times 7\ H_2O$	123
KCl	5.80
$NaHCO_3$	64.8
$Na_2SiO_3 \times 5\ H_2O$	7.47
$NaNO_3$	0.274
KH_2PO_4	0.143
K_2HPO_4	0.184
$Na_2EDTA \times 2\ H_2O$	2.50
$FeSO_4 \times 7\ H_2O$	0.996
H_3BO_3	2.86
$MnCl_2 \times 4\ H_2O$	0.361
LiCl	0.306
$SrCl_2 \times 6\ H_2O$	0.152
RbCl	0.0710
NaBr	0.0160
$Na_2MoO_4 \times 2\ H_2O$	0.0615
$CuCl \times 2\ H_2O$	0.0168
$ZnCl_2$	0.0130
$CoCl_2 \times 6\ H_2O$	0.0100
KI	0.00325
Na_2SeO_3	0.00219
NH_4VO_3	0.000575
Thiaminhydrochloride	0.075
Cyanocobalamin	0.0010
Biotin	0.00075
pH	8.2 ± 0.8

* = original recipe: 293.8 mg/L, modified to achieve a total water hardness of 160 to 180 mg $CaCO_3$/L

4.2 Experimental Procedure

Preparation of the saturated solution	A saturated solution with a nominal loading of 45 mg/L of the test item was freshly prepared prior to the start of the exposure (at 0 hours) and prior to the renewal of the test solutions (at 24 hours). An appropriate amount of the test item as weighed out and transferred into a glass flask with an appropriate amount of the dilution water (Table 2). This solution was stirred with a magnetic stirrer at approximately 1100 rpm for 30 minutes at 30 °C, and thereafter, for further 30 minutes at room temperature. After completion of stirring, the dispersion was allowed to stand for 1 hour for separation of undissolved test item. Thereafter, the saturated solution was removed by siphoning from the approximate center of the water body. The saturated solution was checked via laser beam (Tyndall effect) for undissolved test item (formation of an emulsion). No Tyndall effect was observed in any approach. The saturated solution was used as the highest concentration level and as a stock solution for the preparation of further dilution levels by dilution with dilution water.
Test concentrations	The undiluted saturated solution and further five dilution levels prepared out of the saturated solution in a geometric series with a separation factor of 2.2 by dilution of the saturated solution with dilution water, were tested as follows: 1.94 - 4.27 - 9.39 - 20.7 - 45.5 - 100% of the saturated solution The test item concentrations were selected based on the results of a non-GLP preliminary range finding test. For results, see Annex II.
Control	Dilution water without test item incubated under the same conditions as the test groups.
Test method	The study was performed under semi-static conditions (with a water renewal after 24 hours). Due to the volatility of the test item, the study was performed in a closed system without headspace according to OECD guidance document no. 23, ENV/JM/MONO(2000)6, to reduce contact with air and losses of the test item by evaporation.
Test duration	48 hours
Test vessels / volume	Sealed glass flasks (4.5 (ID) x 9.5 (H) cm) with screw were used and filled up to the top with the test solutions. A test volume of approximately 130 mL was provided in each test vessel.
Dilution water	Same composition as the culture medium (see Table 2)

Number of daphnids and replicates	20 daphnids, divided into 4 replicates, each with 5 daphnids, were used for each concentration level and control.
Age of the daphnids at the start of the exposure	Less than 24 hours old daphnids from a healthy stock were used for the study. Juvenile daphnids were removed from the culture vessels at the latest 24 hours before the start of the exposure and discarded. The juveniles born within the following period of max. 24 hours preceding the exposure were used for the test. No first brood progeny was used for the test.
Acclimatization	Acclimatization of the daphnids was not necessary, because the composition of the dilution water was equivalent to the culture medium.
Application	The test vessels were filled up to the top with the test solutions. There was no headspace in the test vessels. The daphnids were inserted with a small amount of dilution water (start of the exposure) or test solution (water renewal) by pipette. Thereafter, the test vessels were closed immediately with screw caps.
Renewal of the test solutions	The test solutions were renewed after 24 hours. For this purpose, a second set of test vessels were filled up to the top with the freshly prepared test solutions and the daphnids were transferred by pipette (see 'Application'). There was no headspace in the test vessels.
Test temperature (target)	18 - 22 °C, constant within ± 1 °C
Illumination (target)	Diffuse light, light intensity of max. 1500 lx
Photoperiod (target)	16/8 hours light/dark cycle
Feeding	The daphnids were not fed during the study.

4.3 Reference Test

A reference test was conducted as an acute immobilization test (acc. to AQS P 9/2 and OECD 202) in Elendt M4 medium (Table 2) under static conditions with a test duration of 24 hours once per month in order to prove the validity of the test system and test conditions at the test facility. The results of the most recent test are presented in section 7.2.

Reference item	Potassium dichromate p.a. (SIGMA)
Purity	99.0%
Batch number	MKBV0900V
Expiry date	2021-11-25
Test concentrations	1.00 – 2.00 – 4.00 mg/L
Ranges of validity	EC_{50} (24 hours): 0.6 - 2.4 mg/L, according to AQS P 9/2 (clone 5), EC_{50} (24 hours): 0.6 - 2.1 mg/L, according to OECD 202 (clone A)
Exposure phase	2017-11-02 to 2017-11-03

4.4 Type and Frequency of Measurements

4.4.1 Biological Parameters

Immobilization and other observations	Immobilization was determined in all groups after 24 and 48 hours. An animal was considered immobile, if it was not able to swim in the water phase within 15 seconds after gentle agitation of the test vessel. Other adverse effects did not appear.

4.4.2 Water Quality Parameters

Dilution water	Prior to the start of the exposure (0 hours) and the renewal of the test solutions (24 hours), the water quality parameters (i.e. pH-value, dissolved oxygen concentration, temperature, conductivity and total hardness) of the dilution water were measured.
Test media	At the start of the exposure and at the renewal (0 and 24 hours), the water quality parameters of the fresh media (i.e. pH-value, dissolved oxygen concentration) were measured in one additional replicate (without daphnids) per concentration level and control. At the renewal and at the end of the exposure (24 and 48 hours), the water quality parameters of the old media were measured in one appropriate replicate (containing daphnids) per concentration level and control. Preferably the replicate with the highest immobilization rate per concentrations level and control was measured.

Temperature	The incubator temperature (measurement in air with a thermo-hygrograph) was recorded throughout the period of the test.
Criteria for the water quality parameters (target)	• The dissolved oxygen concentration in the 24-hour old media should be ≥ 3 mg/L in all tested concentration levels and in the control. • The pH should be in the range of 6 – 9. • The deviation of the final pH-values (old media) from the initial values (fresh media) should not exceed 1.5 units.

4.4.3 Equipment

Balances	SARTORIUS and KERN
Conductometer	Cond 340i (WTW)
Incubator with Timer	Rumed (RUBARTH APPARATE)
Laser pointer	Laser Lichtzeiger 2316 (KAISER FOTOTECHNIK)
Magnetic stirrer	Variomag Mono (THERMO SCIENTIFIC)
Oximeter and pH-Meter	HQ 40d multi (HACH LANGE)
Piston-stroke pipettes	Finnpipette F2 (THERMO SCIENTIFIC)
Spectrophotometer	DR 5000 (HACH LANGE)
Thermohygrograph	THIES

Standard laboratory equipment

4.4.4 Analytical Monitoring

Determination of the test item	All concentration levels and the control were analytically verified via GC-MS in the fresh media at the start of exposure and at the renewal of the test solutions (0 and 24 hours) and in the 24-hours old media at the renewal and at the end of the exposure (24 and 48 hours). The method was validated prior to this study according to SANCO 3029/99 rev.4 (2000). Details of the analytical method are presented in section 14. Results of the method validation are presented in Annex I. Analytical results are presented in section 7.1.3.
Sampling for the analytical monitoring	At the start of the exposure and at the renewal (0 and 24 hours), samples of the fresh media were taken after preparation of all test item concentrations and analyzed. At the renewal and at the end of the exposure (24 and 48 hours), samples of the 24-hours old media were taken directly from the test vessels and analyzed.
Criteria for the analytical monitoring	Recoveries of the test item should be within ± 20% of the initially measured concentrations.

5 Evaluation

Methods of evaluation

The EC_{100}-values (after 24 and 48 hours) were empirically derived from the observation data. The effect concentrations ($EC_{10\,/\,50\,/\,100}$) were based on the geometric mean measured concentrations of the test item PI 1525.

EC_x-values and statistical analyses

The EC_{10}- and the EC_{50}-values (after 24 and 48 hours of exposure) were calculated by sigmoidal dose-response regression with the software GraphPad Prism.
Since only one partial effect was observed after 24 hours, the highest concentration level without any effects (EC_0) and the lowest concentration level causing 100% immobilization (EC_{100}) were used as 95% confidence limits for the 24-hours $EC_{10\,/\,50}$. The respective 95% confidence limits for the 48-hours $EC_{10\,/\,50}$ were calculated from the standard error and the t-distribution. All calculations were carried out from the best-fit values with the software GraphPad Prism.
The concentration-effect relationships are shown graphically.
The EC_{50}-value for the reference item was calculated by sigmoidal dose-response regression. The respective 95 % confidence limits for the EC_{50} of the reference item were calculated from the standard error and the t-distribution. All calculations were carried out from the best-fit values with the software GraphPad Prism.

Software

All data were computer-processed and rounded for presentation. Consequently, minor variations may occur from the original figures if manual calculations based on the original figures are made subsequently. Calculations were made using the following software:

- GraphPad Prism, GRAPHPAD SOFTWARE, INC.
- Excel, MICROSOFT CORPORATION

6 GLP

Dates

Study initiation	2017-11-03
Experimental starting	2017-11-15
Experimental completion	2017-11-23
Study completion	Please refer to page 1

Chronological test description	- Collection of the juvenile daphnids - Determination of the water quality parameters of the dilution water (at 0 and 24 hours) - Preparation of the saturated solution and the concentration levels by dilution of the saturated solution at 0 hours (experimental starting) and at 24 hours - Determination of the water quality parameters of all concentration levels and the control in the fresh media at the start of the exposure and at renewal (0 and 24 hours) and in old media at renewal and at the end of the test (24 and 48 hours) - Application - Incubation - Determination of the test item concentrations in the fresh media at the start of the exposure and at renewal (0 and 24 hours) and in old media at renewal and at the end of the test (24 and 48 hours) - Water renewal after 24 hours (transfer of the daphnids to the fresh media) - Determination of immobilization after 24 and 48 hours - Evaluation of data
Deviations from the study guideline	None
Deviations from the study plan	None
Archiving	The following will be retained in the archive of the test facility for at least 15 years: • All raw data • Study plan • Final report • All records performed by the quality assurance program including master schedules • Sample of test and reference item

7 Results

7.1 Results of the Definitive Test

7.1.1 Biological Data

The percentage of immobility, determined in all concentration levels of the test item and in the control after 24 and 48 hours is given in Table 3. The absolute numbers of immobile daphnids are presented in Table 4.

Table 3: **Immobilization Rates after 24 and 48 hours of Exposure in the Definitive Test**
(n = 20, divided into 4 replicates with 5 daphnids each)

Geometric mean measured test item concentration [mg/L]	IMMOBILIZATION [%]									
	24 hours					48 hours				
	Replicates					Replicates				
	1	2	3	4	MV	1	2	3	4	MV
13.4	100	100	100	100	100	100% mortality after 24 hours				
8.42	60	0	0	0	15	100	100	100	100	100
4.24	0	0	0	0	0	100	80	100	60	85
1.69	0	0	0	0	0	80	20	20	60	45
0.811	0	0	0	0	0	0	0	0	0	0
0.415	0	0	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0	0	0

Table 4: **Absolute Numbers of immobile Daphnids after 24 and 48 hours of Exposure in the Definitive Test**
(n = 20, divided into 4 replicates with 5 daphnids each)

Geometric mean measured test item concentration [mg/L]	NUMBER OF IMMOBILE DAPHNIDS / TOTAL NUMBER OF DAPHNIDS									
	24 h					48 h				
	Replicates					Replicates				
	1	2	3	4	MV	1	2	3	4	MV
13.4	5 / 5	5 / 5	5 / 5	5 / 5	20 / 20	5 / 5	5 / 5	5 / 5	5 / 5	20 / 20
8.42	3 / 5	0 / 5	0 / 5	0 / 5	3 / 20	5 / 5	5 / 5	5 / 5	5 / 5	20 / 20
4.24	0 / 5	0 / 5	0 / 5	0 / 5	0 / 20	5 / 5	4 / 5	5 / 5	3 / 5	17 / 20
1.69	0 / 5	0 / 5	0 / 5	0 / 5	0 / 20	4 / 5	1 / 5	1 / 5	3 / 5	9 / 20
0.811	0 / 5	0 / 5	0 / 5	0 / 5	0 / 20	0 / 5	0 / 5	0 / 5	0 / 5	0 / 20
0.415	0 / 5	0 / 5	0 / 5	0 / 5	0 / 20	0 / 5	0 / 5	0 / 5	0 / 5	0 / 20
Control	0 / 5	0 / 5	0 / 5	0 / 5	0 / 20	0 / 5	0 / 5	0 / 5	0 / 5	0 / 20

The concentration-effect relationships after 24 and 48 hours of exposure are illustrated graphically in Figure 1 and Figure 2. The effect concentrations ($EC_{10\,/\,50\,/\,100}$), based on the geometric mean measured concentrations of the test item, are presented in Table 1.

7.1.2 Additional Observations during the Definitive Test

All tested concentration levels were visually clear throughout the exposure period. No Tyndall effect was observed in the saturated solution (observations directly after preparation at 0 and 24 hours). No immobility or any adverse effects were observed at the two lowest concentration levels (0.415 and 0.811 mg/L) and in the control.

7.1.3 Measured Exposure Concentrations during the Definitive Test

The concentrations of the test item were analytically verified via GC-MS in the fresh media at the start of the exposure and at the renewal of the test solutions (0 and 24 hours) and in the old media at the renewal and at the end of the test (24 and 48 hours) in all concentration levels and in the control. Details of the analytical method are presented in section 14. Results of the method validation are presented in Annex I.
The measured concentrations in the old media at the renewal and at the end of the test (24 and 48 hours) were in the range of 63 to 128% of the initially measured concentrations. The geometric mean measured concentrations are: 0.415 – 0.811 – 1.69 – 4.24 – 8.42 – 13.4 mg/L. The analytical results are presented in Table 5.

Table 5: **Measured Concentrations of the Test Item PI 1525 during the Definitive Test**

Sampling date	Fresh media, 0 hours	Old media, 24 hours		Fresh media, 24 hours	Old media, 48 hours		**Geometric mean measured test item concentration [mg/L]**
Dilution level of the saturated solution [%]	**PI 1525**						
	Meas. conc. [mg/L]	Meas. conc. [mg/L]	%	Meas. conc. [mg/L]	Meas. conc. [mg/L]	%	
100*	14.3	12.6	88	Not measured due to 100% mortality after 24 hours			**13.4**
45.5	6.85	4.65	68	11.1	14.2	128	**8.42**
20.7	3.04	2.56	84	6.27	6.64	106	**4.24**
9.39	1.31	0.949	73	2.36	2.81	119	**1.69**
4.27	0.615	0.385	63	1.34	1.37	102	**0.811**
1.94	0.366	0.250	68	0.590	0.550	93	**0.415**
Control	< LOQ	< LOQ		< LOQ	< LOQ		

Meas. conc. = measured concentration of the test item, enrichment and dilution factors taken into account
% = percentage of the initially measured concentration of the test item
LOQ = limit of quantification of the *analytical method* (0.002 mg test item/L)
* = saturated solution

7.1.4 Water Quality Parameters

The measured water quality parameters (i.e. pH-value, dissolved oxygen concentration, total water hardness and water temperature) were within the acceptable limits during the study. For results, see Table 7 to Table 9. During the test period, the temperature in the incubator was 19 – 20 °C.

7.2 Test of the Reference Item

The percentage of immobility for the reference item potassium dichromate (SIGMA-ALDRICH, batch number MKBV0900V, purity 99.0%, CAS RN 7778-50-9) was determined after 24 hours from 2017-11-02 to 2017-11-03. For results of the most recent of the monthly performed reference tests, see Table 6.

Table 6: **EC_{50}-Value (with 95% Confidence Limits) of the Reference Item Potassium dichromate** based on nominal concentrations [mg/L], (0 - 24 hours)

	Current Study	Valid Range
EC_{50}	2.10 mg/L	0.6 - 2.4 mg/L, acc. to AQS P 9/2 (02/2000); clone 5 0.6 - 2.1 mg/L, acc. to OECD 202 (2004); clone A
95% confidence limits	1.92 - 2.43 mg/L	

8 Validity Criteria

The study was performed according to OECD Guideline 202 (2004). The validity criteria were fulfilled:

- In the control group, no daphnids were immobilized or showed any signs of disease or stress, e.g. discoloration or unusual behavior such as trapping on the surface of the water, during the 48-hour test period (required: not more than 10% of the daphnids in the control).
- The dissolved O_2 concentration in the 24-hours old media was ≥ 8.11 mg/L (required: ≥ 3 mg/L in the 24-hours old media) in all concentration levels and in the control.

9 Conclusions

Based on the geometric mean measured concentrations of the test item PI 1525, the 48 hours-EC_{50} for *Daphnia magna* was 1.90 mg/L (95% confidence limits: 1.55 – 2.49 mg/L).

10 Literature / References

(1) AQS P 9/2 (02/2000) for daphnids clone 5 cultured in Elendt M4 medium: Bestimmung der nicht akut giftigen Wirkung von Abwasser gegenüber Daphnien über Verdünnungsstufen (DIN 38412 - L 30)

(2) Directive 2004/10/EC, The OECD Principles of Good Laboratory Practice (GLP)

(3) OECD-Guideline 202 for Testing of Chemicals (adopted 13. April 2004): Daphnia sp., Acute Immobilization Test

(4) OECD (2000): Guidance document on aquatic toxicity testing of difficult substances and mixtures. OECD series on testing and assessment no. 23, ENV/JM/MONO(2000)6

(5) OECD Principles on Good Laboratory Practice (as revised in 1997), ENV/MC/Chem(98)17, Environment Directorate, OECD, Paris, 1999

(6) Principles of Good Laboratory Practice – German Chemical Law (ChemG), Annex 1

(7) SANCO/3029/99 rev.4, Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414 (11/07/00)

11 Graphs: Results of Statistical Analysis

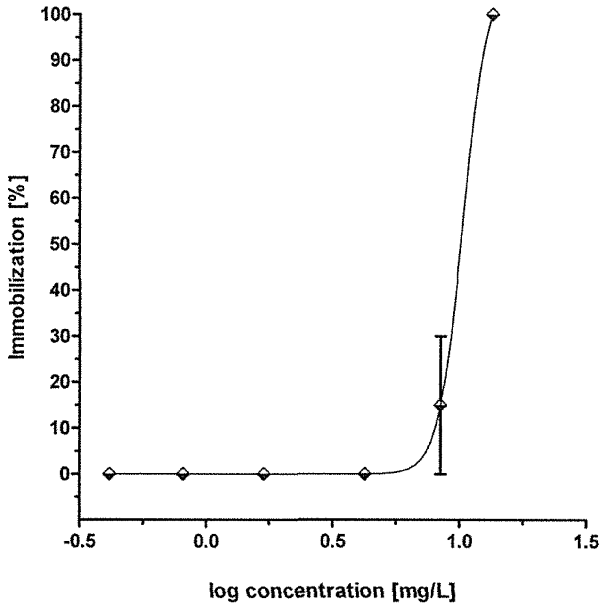


Figure 1: **Concentration-Effect Relationship of the Test Item PI 1525 after 24 hours**
(based on the geometric mean measured concentrations [mg/L])

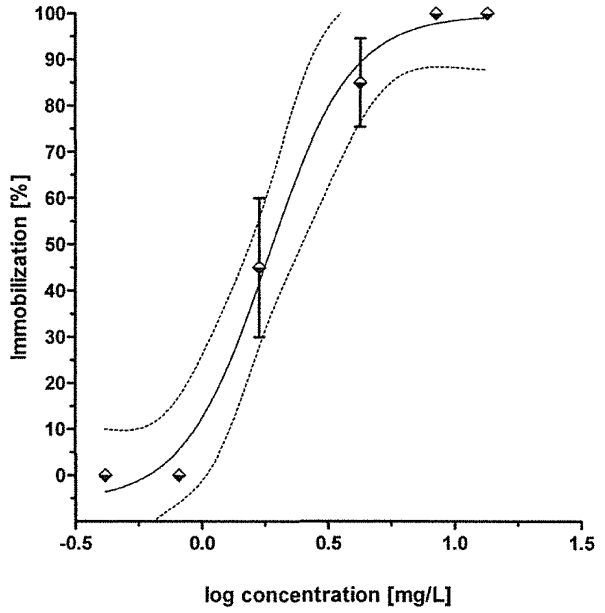


Figure 2: **Concentration-Effect Relationship of the Test ItemPI 1525 after 48 hours**
(based on the geometric mean measured concentrations [mg/L])

12 Physicochemical Data

Table 7: **Water Quality Parameters in the fresh Media at the Start of the Exposure and at the Renewal (0 and 24 hours)**
(measured in one additional replicate (without daphnids) per concentration level and control)

Geometric mean measured test item concentration [mg/L]	0 hours		24 hours	
	pH-value	Dissolved O_2 concentration [mg/L]	pH-value	Dissolved O_2 concentration [mg/L]
13.4	7.74	9.06	Not determined, due to 100% mortality after 24 hours	
8.42	7.78	9.19	7.53	8.88
4.24	7.76	9.19	7.52	8.96
1.69	7.76	9.22	7.48	9.03
0.811	7.79	9.21	7.48	8.98
0.415	7.85	9.22	7.57	8.73
Control	7.79	9.27	7.49	8.86

Table 8: **Water Quality Parameters in the 24-hours old Media at the Renewal and at the End of the Exposure (24 and 48 hours)**
(measured in one replicate (containing daphnids) with the highest immobilization rate per concentration level and control)

Geometric mean measured test item concentration [mg/L]	24 hours			48 hours		
	pH-value	Dissolved O_2 concentration [mg/L]	Replicate number	pH-value	Dissolved O_2 concentration [mg/L]	Replicate number
13.4	7.49	8.11	1	Not determined, due to 100% mortality after 24 hours		
8.42	7.52	8.12	1	7.41	8.44	1
4.24	7.54	8.20	1	7.42	8.41	1
1.69	7.54	8.55	1	7.44	8.76	1
0.811	7.56	8.73	1	7.48	8.98	1
0.415	7.41	8.99	1	7.46	8.56	1
Control	7.37	8.93	1	7.53	8.36	1

Table 9: **Water Quality Parameters of the Dilution Water at the Start of the Exposure and at the Renewal (0 and 24 hours)**

Dilution water dated:	pH-Value	Dissolved O_2 concentration [mg/L]	Temperature [°C]	Conductivity [µS/cm]	Total hardness [mg $CaCO_3$/L]
0 hours: 2017-11-15	7.99	9.23	20.7	423	166
24 hours: 2017-11-16	7.73	9.28	20.7	487	175

13 Statistics

13.1 Evaluation of the EC_x-Values after 24 hours based on the Geometric Mean Measured Concentrations of the Test Item

Equation: Sigmoidal dose-response, variable slope
Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))

Log concentration [mg/L]	Immobilization after 24 hours [%]			
-0.382	0	0	0	0
-0.091	0	0	0	0
0.228	0	0	0	0
0.627	0	0	0	0
0.925	60	0	0	0
1.127	100	100	100	100

Fitting Results

Sigmoidal dose-response (variable slope)	Results of the fitting curve, based on the immobilization rates [%] after 24 hours per concentration level [mg/L]
Best-fit values	Ambiguous
Bottom	-0.006854
Top	~ 107.8
LogEC50	~ 1.010
HillSlope	~ 9.399
EC10 [mg/L]	8.020
EC20 [mg/L]	8.733
EC50 [mg/L]	10.06
Std. error	
Bottom	3.355
Top	~ 1422
LogEC50	~ 3.903
HillSlope	~ 357.3
95% Confidence limits	
Bottom	-7.004 to 6.991
Top	(Very wide)
LogEC50	(Very wide)
HillSlope	(Very wide)
EC10 [mg/L]	4.134 to 13.34
EC20 [mg/L]	4.505 to 13.34
EC50 [mg/L]	8.449 to 13.37
Goodness of fit	
Degrees of freedom	20
R square	0.9224
Absolute sum of squares	2700
Sy.x	11.62
Number of points	
Analysed	24

Sy.x = standard deviation of the residuals, expressed in the same units as y
Hillslope = equivalent to slope

13.2 Evaluation of the EC_x-Values after 48 hours based on the Geometric Mean Measured Concentrations of the Test Item

Equation: Sigmoidal dose-response, variable slope
Y=Bottom + (Top-Bottom)/(1+10^((LogEC50-X)*HillSlope))

Log concentration [mg/L]	Immobilization after 48 hours [%]			
-0.382	0	0	0	0
-0.091	0	0	0	0
0.228	80	20	20	60
0.627	100	80	100	60
0.925	100	100	100	100
1.127	100	100	100	100

Fitting Results

Sigmoidal dose-response (variable slope)	Results of the fitting curve, based on the immobilization rates [%] after 48 hours per concentration level [mg/L]
Best-fit values	
Bottom	-5.723
Top	99.69
LogEC50	0.2606
HillSlope	2.628
EC10 [mg/L]	0.9393
EC20 [mg/L]	1.185
EC50 [mg/L]	1.903
Std. error	
Bottom	8.848
Top	6.192
LogEC50	0.05681
HillSlope	1.009
95% Confidence limits	
Bottom	-24.18 to 12.74
Top	86.77 to 112.6
LogEC50	0.1421 to 0.3791
HillSlope	0.5235 to 4.733
EC10 [mg/L]	0.5572 to 1.291
EC20 [mg/L]	0.8776 to 1.507
EC50 [mg/L]	1.552 to 2.493
Goodness of fit	
Degrees of freedom	20
R square	0.9147
Absolute sum of squares	4112
Sy.x	14.34
Number of points	
Analysed	24

Sy.x = standard deviation of the residuals, expressed in the same units as y
Hillslope = equivalent to slope

14 GC-MS Analysis of PI 1525

14.1 Method

Method of determination	Analytical evaluation of the test item concentrations and the control was carried out via SPME-GC-MS on a TG 5-MS capillary column with SPME using the test item for external standard calibration. The evaluation was performed as a group evaluation of two isomers, the detection was performed with a mass selective detector (MS).

GC-MS-System, compiled of:

GC	CP-3800, VARIAN
Autosampler	Combi PAL with SPME option, CTC ANALYTICS
Detection	MS, Saturn 2000, VARIAN
Software	MS Workstation 6.8 (SP1), VARIAN
Analytical Column	TG 5-MS, 30 m, 0.25 mm ID, 0.25 µm film thickness, batch: 1113387 THERMO SCIENTIFIC
Inlet liner	Skyliner SPME, 0.75 * 5.0 * 54 mm, batch: 870195BL, RESTEK
SPME fiber	100 µm PDMS, batch: 87076, SUPELCO
Additional equipment	Positive-displacement pipettes, GILSON MEDICAL
Reagents	Acetone, ≥ 99.7%, VWR Demineralized water (A. demin) (in-house device SG Series compact, SG WATER) Sodium chloride, > 99.8%, ROTH
Analytical standard	The test item was used as external standard.

CONDITIONS OF SPME

Agitator temperature	50 °C
Pre incubation time	5 min
Extraction time	10 min
Desorption time	2 min

CONDITIONS OF ANALYSIS

Carrier gas	Helium 1.0 mL /min
Retention time	approx. 9.3 (group of 2 peaks)
Injector	Splitless for 2.0 min
Injector temperature	250 °C

GC Oven program

Table 10: **GC Temperature program**

Temperature [°C]	Heating rate [°C/min]	Hold time [min]
50	0	1.0
100	20	2.0
200	10	2.5
250	25	2.0

CONDITIONS OF DETECTION

Ionisation mode	Electron Impact (EI)
Ion polarity	Positive
Scan mode	Centroid
Scan method	Full scan (40-250 m/z)
Scan time	0.5 sec
Detector temperature	220°C
Quantification ions [m/z]	91, 106, 119, 134 (quantification based on the sum of the respective responses)

Preparation of the standards A stock solution of 25000 mg/L test item in acetone was first diluted to 1000 mg/L and then to 7 concentrations with acetone. 100 µL of each standard solution was pipetted into a 20 mL headspace vial containing 9.9 mL NaCl solution (100 g NaCl/L A. demin). For the calibration range using the above standards, see section 15.2.1.

Preparation of the samples

All test item concentrations were first diluted with acetone and in the last dilution step with NaCl solution (100 g NaCl/L A. demin) prior to analysis. The control was only diluted with NaCl solution. The last dilution step took place in a 20 mL headspace vial. For details refer to Table 11.

Table 11: **Dilution steps for the first exposure interval**

Saturated solution [%]	Dilution Factor	Sample volume [mL]	Final volume [mL]
100	100000	0.01 [1)] 0.1 [2)]	10 [1)] 10 [2)]
45.5	50000	0.02 [1)] 0.1 [2)]	10 [1)] 10 [2)]
20.7	20000	0.05 [1)] 0.1 [2)]	10 [1)] 10 [2)]
9.39	10000	0.1 [1)] 0.1 [2)]	10 [1)] 10 [2)]
4.27	5000	0.2 [1)] 0.1 [2)]	10 [1)] 10 [2)]
1.94	1000	1.0 [1)] 0.1 [2)]	10 [1)] 10 [2)]
Control	5	2.0 [3)]	10 [3)]

1) First dilution step with acetone
2) Second dilution step with NaCl solution
3) Dilution step with NaCl solution

Table 12: **Dilution steps for the second exposure interval**

Saturated solution [%]	Dilution Factor	Sample volume [mL]	Final volume [mL]
100	-	-	-
45.5	25000	0.04 [1)] 0.1 [2)]	10 [1)] 10 [2)]
20.7	10000	0.1 [1)] 0.1 [2)]	10 [1)] 10 [2)]
9.39	5000	0.2 [1)] 0.1 [2)]	10 [1)] 10 [2)]
4.27	2500	0.4 [1)] 0.1 [2)]	10 [1)] 10 [2)]
1.94	500	1.0 [1)] 0.1 [2)]	5 [1)] 10 [2)]
Control	5	2.0 [3)]	10 [3)]

1) First dilution step with acetone
2) Second dilution step with NaCl solution
3) Dilution step with NaCl solution

Sample storage

All samples were stored at 6 ± 2 °C until sample preparation and at room temperature until the start of the analysis (on an autosampler), if necessary.

Evaluation

Quantification of the test item was calculated by peak area (group evaluation) based on the external standard.

14.2 Representative Calibration Curve

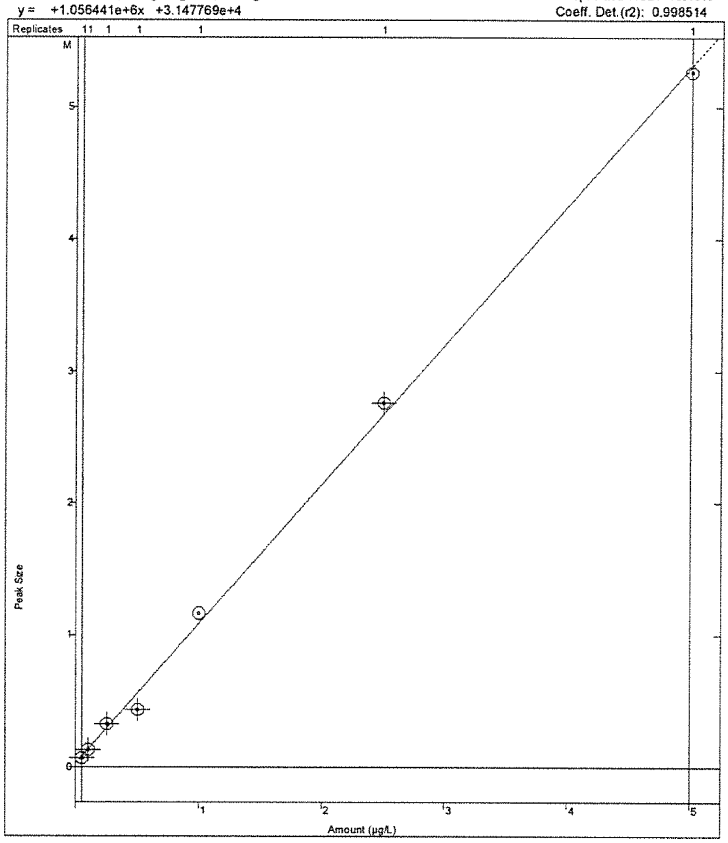
Calibration Curves Report 16.11.2017 18:18

Method:	...27sy\171115\dai171971_a0_group_spmered_promth_171115.mth		
Recalc Method:	...mth_171115.mth	Last Calibration:	16.11.2017 16:10
Sample List:	N/A	Cmpd. Table Updated:	16.11.2017 16:10
Sequence:	N/A	Detector:	2000 Mass Spec
MS Workstation (Upgrade)		Workstation Version:	Version 6.8
Peak Measurement:	Area	Calibration Type:	External Standard Analysis

PI1525_group

Curve Fit: Linear, Origin: Ignore, Weight: None
y = +1.056441e+6x +3.147769e+4

Resp. Fact. RSD: 15.40%
Coeff. Det.(r2): 0.998514



Ret. Time:	9.214 min.	Peak Name:	PI1525_group	
Level	Amount	Replicate No.	Response	Avg. Response
1	0.050000	1	69049	69049.5
2	0.100000	1	131848	131848.2
3	0.250000	1	326084	326083.9
4	0.500000	1	433031	433031.0
6	1.000000	1	1164599	1164599.3
7	2.500000	1	2760483	2760482.8
8	5.000000	1	5265797	5265796.5

Figure 3: **Calibration Curve of the Standard (Group evaluation)**
(dated 2017-11-15/16)

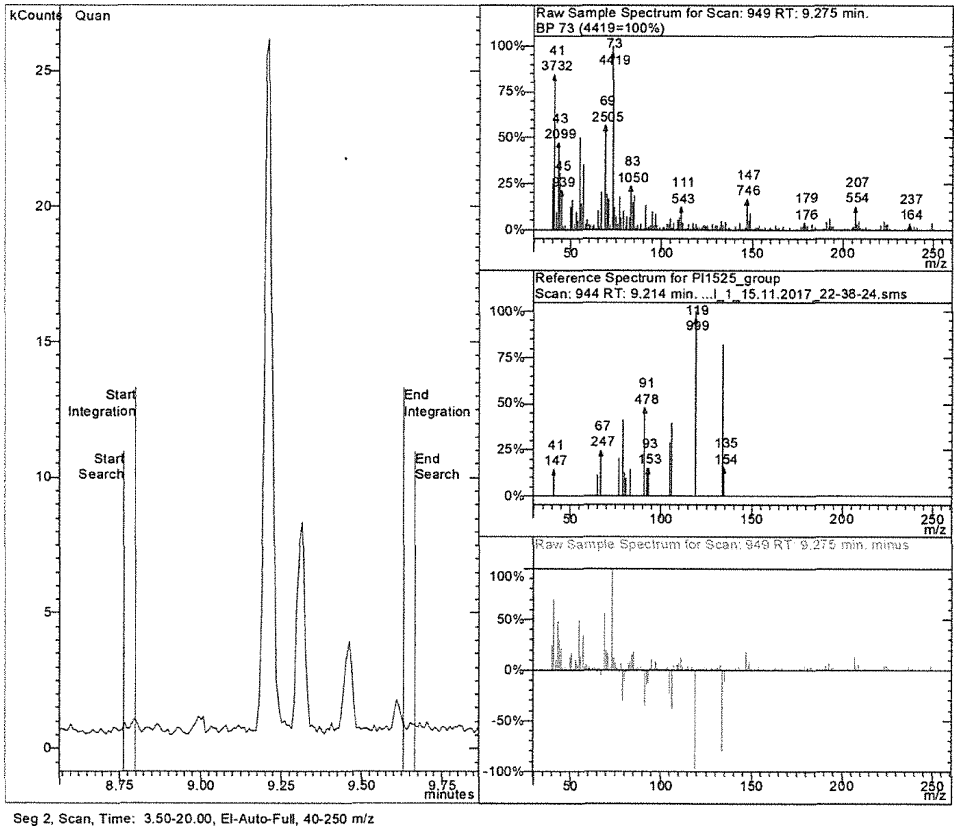
14.3 Representative Chromatograms

Target Compound Report for #1 from ...I_1_15.11.2017_18-31-34.sms

Sample ID:	17197_0,05ugL_1	Operator:	Da
Instrument ID:		Last Calibration:	16.11.2017 16:08
Measurement Type:	Area	Calibration Type:	External Standard
Acquisition Date:	15.11.2017 18:31	Data File:	...11.2017_18-31-34.sms
Calculation Date:	16.11.2017 16:08	Method:	...ed_promth_171115.mth
Sample Type:	Calibration		
Inj. Sample Notes:	170927SY; DAI171971;		
Divisor:	1.000000	Multiplier:	1.000000

Parameter	Specification	Actual	Status
Compound Number		1	
Retention Time	9.214 +/- 0.450	9.278 min.	Pass
RT Offset		Δ0.064 min.	
Peak Name		PI1525_group	
CAS Number		None	
Quan Ions	91.0+106.0+119.0+134.0		
Area	>=200	69049	Pass
Height		25677	
Amount (RF)		1.381e+6	
Reverse Match		N/A	
Calibration Equation	Linear, Ignore, None	y = +1.0809e+6x +5.3672e+4	
Baseline Code		GR	
Status		U	Pass
Error		None	

Status and Errors: U : User-defined EndPoints.



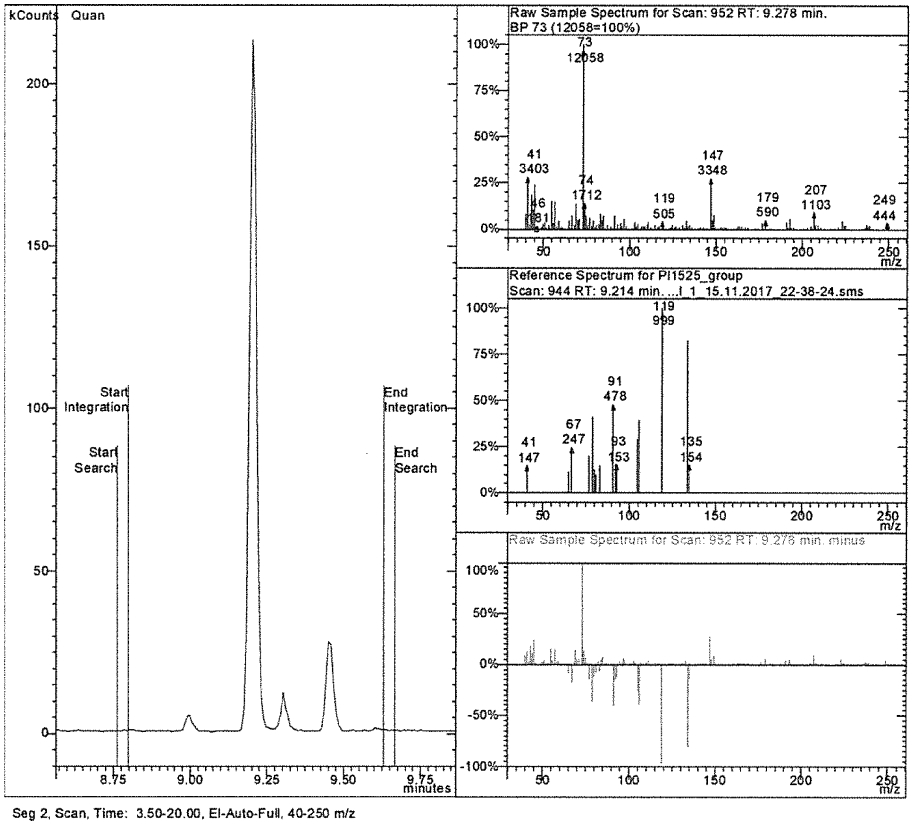
Seg 2, Scan, Time: 3.50-20.00, EI-Auto-Full, 40-250 m/z

Figure 4: **Chromatogram of the Lowest Standard**
0.05 µg/L (dated 2017-11-15)

Target Compound Report for #1 from ...94%_15.11.2017_21-05-47.sms

Sample ID:	DAI171971_A0_1,94%	Operator:	Da
Instrument ID:		Last Calibration:	16.11.2017 16:10
Measurement Type:	Area	Calibration Type:	External Standard
Acquisition Date:	15.11.2017 21:05	Data File:	...11.2017_21-05-47.sms
Calculation Date:	16.11.2017 16:10	Method:	...ed_promth_171115.mth
Sample Type:	Analysis		
Inj. Sample Notes:	170927SY; DAI171971;		
Divisor:	1.000000	Multiplier:	1.000000

Parameter	Specification	Actual	Status
Compound Number		1	
Retention Time	9.214 +/- 0.450	9.278 min.	Pass
RT Offset		Δ0.064 min.	
Peak Name		PI1525_group	
CAS Number		None	
Quan Ions	91.0+106.0+119.0+134.0		
Area	>=200	418121	Pass
Height		256477	
Amount	>= 0.000 µg/L	0.366 µg/L	Pass
Reverse Match		N/A	
Calibration Equation	Linear, Ignore, None	y = +1.0564e+6x +3.1478e+4	
Baseline Code		GR	
Status			Pass
Error		None	



Seg 2, Scan, Time: 3.50-20.00, EI-Auto-Full, 40-250 m/z

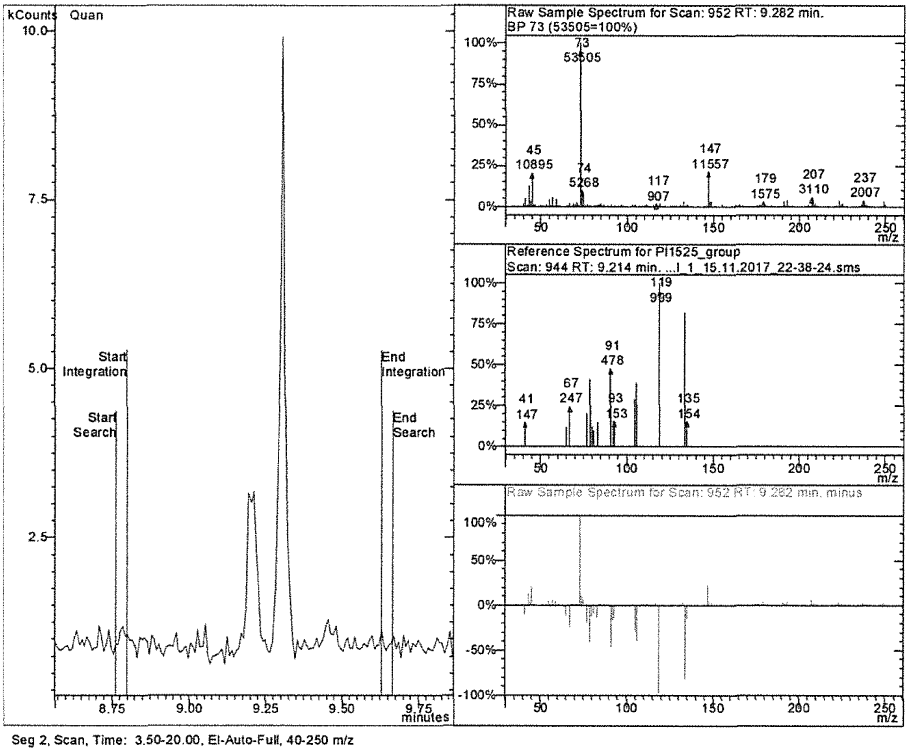
Figure 5: **Chromatogram of the Test Item in fresh Medium at the Start of the Exposure (0 hours)**
1.94% of the saturated solution, dilution factor 1000 (dated 2017-11-15)

Target Compound Report for #1 from ...r_i_15.11.2017_20-04-06.sms

Sample ID:	DAI171971_A0_Ktr_I	Operator:	Da
Instrument ID:		Last Calibration:	16.11.2017 16:10
Measurement Type:	Area	Calibration Type:	External Standard
Acquisition Date:	15.11.2017 20:04	Data File:	...11.2017_20-04-06.sms
Calculation Date:	16.11.2017 16:10	Method:	...ed_promth_171115.mth
Sample Type:	Analysis		
Inj. Sample Notes:	170927SY; DAI171971;		
Divisor:	1.000000	Multiplier:	1.000000

Parameter	Specification	Actual	Status
Compound Number		1	
Retention Time	9.214 +/- 0.450	9.278 min.	Pass
RT Offset		Δ0.064 min.	
Peak Name		PI1525_group	
CAS Number		None	
Quan Ions	91.0+106.0+119.0+134.0		
Area	>=200	11792	Pass
Height		9101	
Amount	>= 0.000 µg/L	N/A	Fail
Reverse Match		N/A	
Calibration Equation	Linear, Ignore, None	y = +1.0564e+6x +3.1478e+4	
Baseline Code		GR	
Status		X *C	Error
Error		* C	

Status and Errors: X : Error
* : Negative or imaginary result. Check calibration curve.
C : Result out of Tolerance or Calibration Range.



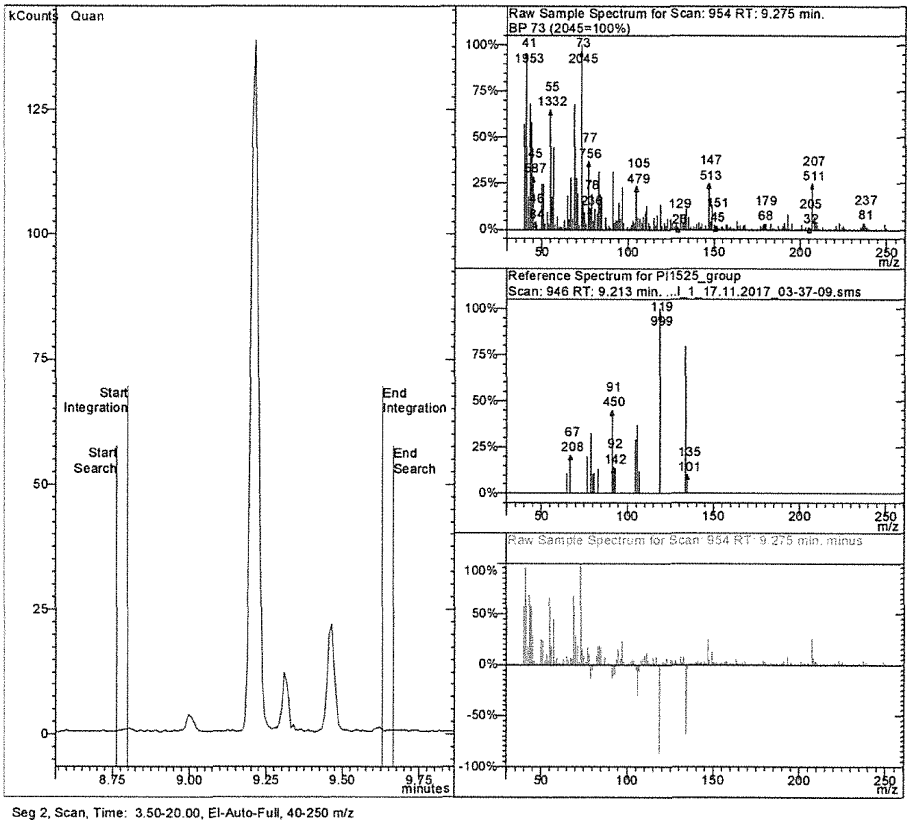
Seg 2, Scan, Time: 3.50-20.00, EI-Auto-Full, 40-250 m/z

Figure 6: **Chromatogram of the Control in Fresh Medium at the Start of the Exposure (0 hours)** < LOQ, dilution factor 5 (dated: 2017-11-15)

Target Compound Report for #1 from ...94%_16.11.2017_20-56-10.sms

Sample ID:	DAI171971_E0_1,94%	Operator:	Da
Instrument ID:		Last Calibration:	17.11.2017 13:53
Measurement Type:	Area	Calibration Type:	External Standard
Acquisition Date:	16.11.2017 20:56	Data File:	...11.2017_20-56-10.sms
Calculation Date:	17.11.2017 13:53	Method:	...ed_promth_171116.mth
Sample Type:	Analysis		
Inj. Sample Notes:	170927SY; DAI171971;		
Divisor:	1.000000	Multiplier:	1.000000

Parameter	Specification	Actual	Status
Compound Number		1	
Retention Time	9.214 +/- 0.450	9.278 min.	Pass
RT Offset		Δ0.064 min.	
Peak Name		PI1525_group	
CAS Number		None	
Quan Ions	91.0+106.0+119.0+134.0		
Area	>=200	281631	Pass
Height		170271	
Amount	>= 0.000 µg/L	0.250 µg/L	Pass
Reverse Match		N/A	
Calibration Equation	Linear, Ignore, None	y = +8.1836e+5x +7.7002e+4	
Baseline Code		GR	
Status			Pass
Error		None	



Seg 2, Scan, Time: 3.50-20.00, EI-Auto-Full, 40-250 m/z

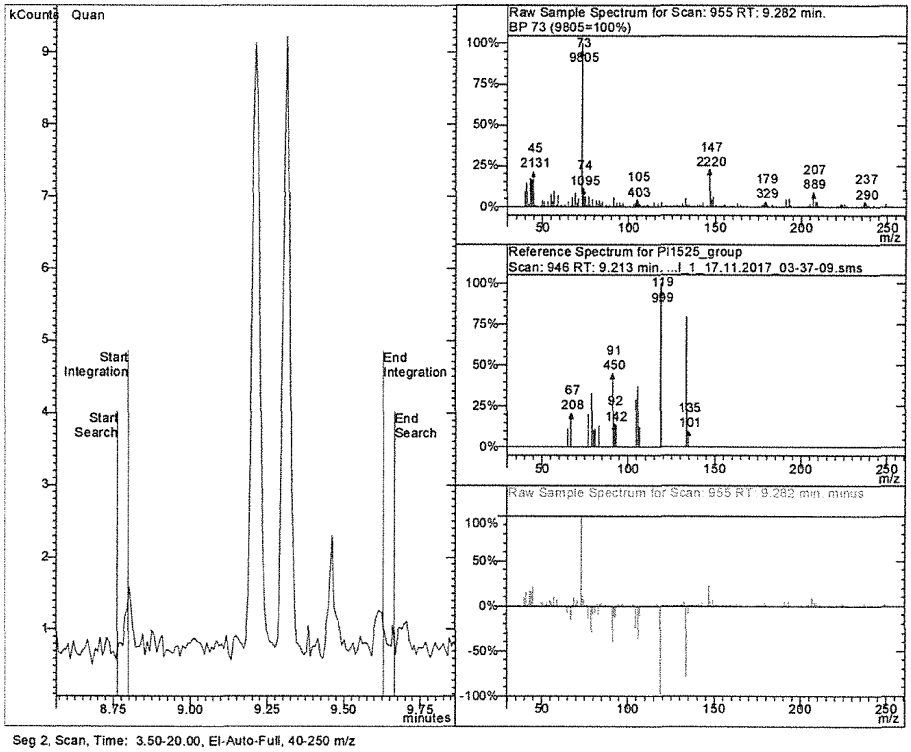
Figure 7: **Chromatogram of the Test Item in old Medium at Renewal (24 hours)**
1.94% of the saturated solution, dilution factor 1000 (dated 2017-11-16)

Target Compound Report for #1 from ...r_i_16.11.2017_20-25-19.sms

Sample ID:	DAI171971_E0_Ktr_I	Operator:	Da
Instrument ID:		Last Calibration:	17.11.2017 13:53
Measurement Type:	Area	Calibration Type:	External Standard
Acquisition Date:	16.11.2017 20:25	Data File:	...11.2017_20-25-19.sms
Calculation Date:	17.11.2017 13:53	Method:	...ed_promth_171116.mth
Sample Type:	Analysis		
Inj. Sample Notes:	170927SY; DAI171971;		
Divisor:	1.000000	Multiplier:	1.000000

Parameter	Specification	Actual	Status
Compound Number		1	
Retention Time	9.214 +/- 0.450	9.278 min.	Pass
RT Offset		Δ0.064 min.	
Peak Name		PI1525_group	
CAS Number		None	
Quan Ions	91.0+106.0+119.0+134.0		
Area	>=200	28463	Pass
Height		16858	
Amount	>= 0.000 µg/L	N/A	Fail
Reverse Match		N/A	
Calibration Equation	Linear, Ignore, None	y = +8.1836e+5x +7.7002e+4	
Baseline Code		GR	
Status		X *C	Error
Error		* C	

Status and Errors:
X : Error
* : Negative or imaginary result. Check calibration curve.
C : Result out of Tolerance or Calibration Range.



Seg 2, Scan, Time: 3.50-20.00, EI-Auto-Full, 40-250 m/z

Figure 8: **Chromatogram of the Control in old Medium at Renewal (24 hours)**
< LOQ, dilution factor 5 (dated: 2017-11-16)

15 Certificate of Test Item Analysis

IDENTITY-CERTIFICATE

==========

Test-Code: PI 1525

Cas-No. : 1897392-68-5 and 13213-08-6

Chemical Name: Mixture of 4,7-Methano-1H-indene, 5-ethoxyoctahydro-,(3aR,4R,5S,7R,7aR)-rel- and 4,7-Methano-1H-indene, 5-ethoxyoctahydro-,(3aR,4S,5R,7S,7aR)-rel-

Batch-No.: Ho 154 262 MM + 0.1% Vit.E.

Appearance/colour: Liquid/clear

Expiry date: 2017 Dec.

Purity: 95.3%
Constituent 1: 4,7-Methano-1H-indene, 5-ethoxyoctahydro-,(3aR,4R,5S,7R,7aR)-rel-, CAS: 1897392-68-5, ca. 83%
Constituent 2: 4,7-Methano-1H-indene, 5-ethoxyoctahydro-,(3aR,4S,5R,7S,7aR)-rel-, CAS: 13213-08-6, ca. 12%

Storage conditions: Store in a tightly closed container at room temperature away from light and moisture.

16 GLP-Certificate of Noack Laboratorien GmbH

Gewerbeaufsicht in Niedersachsen

Staatliches Gewerbeaufsichtsamt Hildesheim

Gute Laborpraxis / Good Laboratory Practice

GLP-Bescheinigung / Statement of GLP Compliance

(gemäß / according to § 19 b Abs.1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

☒ Prüfeinrichtung / Test facility ☐ Prüfstandort / Test site

Noack Laboratorien GmbH Käthe-Paulus-Str. 1 31157 Sarstedt DEUTSCHLAND	**Noack Laboratorien GmbH** Käthe-Paulus-Str. 1 31157 Sarstedt GERMANY

Prüfungen nach Kategorien / Areas of Expertise (gemäß / according ChemVwV-GLP Nr. 5.3/OECD guidance)

1 - Prüfungen zur Bestimmung der physikalisch-chemischen Eigenschaften und Gehaltsbestimmungen	1 - physical-chemical testing
4 - Ökotoxikologische Prüfungen zu Bestimmung der Auswirkungen auf aquatische und terrestrische Organismen	4 - environmental toxicity studies on aquatic and terrestrial organisms
5 - Prüfungen zum Verhalten im Boden, im Wasser und in der Luft, Prüfungen zur Bioakkumulation und zur Metabolisierung	5 - studies on behaviour in water, soil and air; bioaccumulation
6 - Prüfungen zur Bestimmung von Rückständen	6 - residue studies

Ort / Place	Datum der Inspektion / Date of Inspection (Tag.Monat.Jahr / month.day.year)
Sarstedt	**07. – 10. Juni 2016 & 13. Juli 2016 /**
Sarstedt	**Jun 07th – Jun 10th, 2016 & Jul 13th, 2016**

Die/Der genannte Prüfeinrichtung/Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

The above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Hildesheim, 03.01.2017

Staatliches Gewerbeaufsichtsamt Hildesheim
Im Auftrage

Bahn

17 Annex I: Method validation (non-GLP)

17.1 Method Validation

The method showed acceptable results for linearity, accuracy, precision and specificity according to SANCO 3029/99 rev.4 (2000) and fulfilled all study plan requirements. Acceptance criteria and results of the method validation parameter are shown in Table 13 and Table 15.

Table 13: **Parameter, Acceptance Criteria and Results of the Method Validation**

Parameter	Acceptance criteria	Result	
Linearity	5 standard concentrations, $r^2 \geq 0.992$	0.05 to 5 µg /L (n = 7) $r^2 > 0.992$	✓
Lowest calibration standard	S/N ≥ 9 for the signal of ion used for evaluation	0.05 µg/L, S/N 280 (main component)	✓
Limit of Detection (LOD)	S/N of ≥ 3 for quantifier ion trace (not necessary if S/N ≥ 30)	Not determined (S/N lowest calibration level > 30 for main component)	✓
Limit of Quantification (LOQ)	At least 20% above lowest calibration standard after sample preparation	2 µg test item/L (1 x LOQ) 25 mg test item/L (12500 x LOQ)	✓
Accuracy (Fortified samples)	Mean recovery rate of 70-110% (ideally 80-100%) per fortification level (2 levels)	*Daphnia* dilution water : 1 x LOQ: 79% (n = 5) 12500 x LOQ: 89% (n = 5)	✓
Precision	Relative standard deviation ≤ 20% per fortification level	1 x LOQ: 11% 12500 x LOQ: 13%	✓
Specificity (GC-MS)	Analyses with MS (mass-spectrometric detection) as selective detector of at least three specific ions (ideally with an *m/z* ratio ≥ 100). Comparison of spectra of sample peaks against spectra obtained from standard peaks	Quantification ions [m/z]: 91, 106, 119, 134 (quantification will be based on the sum of the respective responses)	✓
	Blank values < 30% of LOQ	Blank values < 30% of LOQ	

✓ criterion fulfilled

17.2 Preparation of the fortified samples

Fortified samples were prepared in *daphnia* dilution medium. For dilution factors, please refer to Table 14.

Table 14: **Preparation of Fortified Samples**

LOQ Level	Control	1	12500
Stock solution [mg test item/L]	-	25000	
Medium	-	Acetone	
Spiking solution	-	0.2 mg test item/L (acetone)	2500 mg test item/L (acetone)
Replicates	2	5	5
Concentration of the LOQ level [µg test item/L]	-	2	25000
Medium for preparation	*Daphnia* dilution water	*Daphnia* dilution water	*Daphnia* dilution water
Volume of spiking solution [mL]	-	0.1	0.1
Volume of medium [mL]	10 [3)]	9.9	9.9
Dilution factor	-	-	25000
First dilution medium			Acetone
Sample volume [mL]			0.1 [1)] 0.4 [2)]
Finale volume [mL]			10 [1)] 1 [2)]
Second dilution medium	NaCl solution [3)]	NaCl solution [3)]	NaCl solution [3)]
Sample volume [mL]	2	2	0.1
Finale volume [mL]	10	10	10

1) First dilution step 2) Second dilution step
3) NaCl solution: 100 g NaCl/L A. demin

17.3 Accuracy and Precision

Table 15: **Measured Concentrations and Percent of the Fortified Samples of PI 1525**
Fortified concentrations *): 1.998 µg/L (1 x LOQ) and 24980 µg/L (12500 x LOQ) of the test item

	PI 1525			
	Daphnia dilution water			
	1 x LOQ		12500 x LOQ	
	Meas. conc. [µg/L]	%	Meas. conc. [µg/L]	%
1	1.47	74	20858	84
2	1.51	76	27228	109
3	1.87	94	21458	86
4	1.60	80	20309	81
5	1.45	73	21683	87
Mean	1.58	79	22307	89
SD	0.2		2803	
CV [%]	11		13	

* = weighing factor taken into account
Meas. conc. = measured concentration of the test item, enrichment and dilution factor taken into account
% = percent of nominal of the fortified concentration
SD = Standard deviation
CV = Coefficient of variation

18 Annex II: Preliminary Range Finding Test (non-GLP)

A non-GLP preliminary range finding test under semi-static conditions over a period of 48 hours was conducted at the test facility with a saturated solution of the test item at a loading of 45 mg/L and two further dilution levels prepared by dilution of the saturated solution by factor 10 and 100 with dilution water (Table 2). The preliminary range finding test was conducted under diffuse light conditions (light intensity of max. 1500 lx, 16/8 hours light/dark cycle).
The saturated solution was freshly prepared prior to the start of the exposure (at 0 hours) and prior to the renewal of the test solutions (at 24 hours) as specified in section 4.2 for the definitive test. The saturated solution and the dilution levels 1 and 10% of the saturated solution were visually clear throughout the exposure period. A Tyndall effect was observed in all test item solutions.
In the range finding test, two replicates per dilution level and control, each with ten daphnids, were tested. The results are presented in Table 16 and Table 17.

Table 16: **Immobilization Rates in the non-GLP Preliminary Range Finding Test**
(n = 20, divided into 2 replicates with 10 daphnids each)

Dilution level of the saturated solution [%]	IMMOBILIZATION [%]					
	24 hours			48 hours		
	Replicates			Replicates		
	1	2	MV	1	2	MV
100*	100	100	100	100% mortality after 24 hours		
10	0	0	0	10	20	15
1	0	0	0	0	0	0
Control	0	0	0	0	0	0

* = saturated solution

Table 17: **Measured Concentrations of PI 1525 during the non-GLP Preliminary Range Finding Test**

Sampling date	Fresh media, 0 hours	Old media, 24 hours		Fresh media, 24 hours	Old media, 48 hours	
Dilution level of the saturated solution [%]	**PI 1525**					
	Meas. conc. [mg/L]	Meas. conc. [mg/L]	%	Meas. conc. [mg/L]	Meas. conc. [mg/L]	%
100*	33.0	25.6	78	42.9	No measurement[2)]	
10	3.50	2.70	77	3.20	2.23	70
1	0.311	0.348	112	0.311	0.261	84
Control	< 0.05[1)]	< 0.05[1)]		< 0.05[1)]	< 0.05[1)]	

* = saturated solution
Meas. conc. = measured concentration of the test item, single determinations, dilution factors taken into account
% = percent of the initially measured concentration of the test item
1) = lowest calibration Level (0.05 mg/L of the test item)
2) = due to 100% mortality after 24 hours